

# Resolution of bovine brain calcineurin subunits: stimulatory effect of subunit B on subunit A phosphatase activity

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Calcineurin was dissociated into subunits A and B by SDS and the dissociated subunits were separated by Sephadex G-100 column chromatography in SDS. The phosphatase activity was associated with the A subunit and was detected only in the presence of  $MnCl_2$  of the various divalent cations tested. The  $Mn^{2+}$ -dependent phosphatase of A subunit was stimulated (4–5-fold) by calmodulin. The subunit B increased only modestly  $Mn^{2+}$  stimulated phosphatase activity of subunit A but markedly increased it when assay also contained calmodulin. These results support the view that subunit B plays an important role in  $Mn^{2+}$ /calmodulin regulation of subunit A phosphatase activity. They also lend further support to our earlier postulate ([1984] FEBS Lett. 169, 251–255) that  $Mn^{2+}$  is a powerful regulator of calcineurin phosphatase.

*Calcineurin    Calcineurin subunit    Protein phosphatase    Calmodulin    Divalent cation*

## 1. INTRODUCTION

Bovine brain calcineurin [1,] a  $Ca^{2+}$ -calmodulin regulated protein phosphatase [2–6], is a heterodimeric protein comprised of subunit A ( $M_r \sim 61\,000$ ) and subunit B ( $M_r \sim 19\,000$ ) [1]. Subunit A was recently shown to contain catalytic region [6,7], binding sites for divalent metals like  $Mn^{2+}$  [5–7] and  $Ni^{2+}$  [8] besides earlier evidence [9] that calmodulin binds to this subunit. Subunit B has been known to bind 4 mol  $Ca^{2+}$  [1] and is apparently devoid of any enzymatic (i.e. phosphatase) activity [6,7]. The role(s) of subunit B in the phosphatase activity of the holoenzyme and specifically in the actions of divalent metals ( $\pm$  calmodulin) remains to be clarified.

We undertook the study of resolved subunits of calcineurin with the aim of examining the actions of B subunit on divalent metal effects on subunit A phosphatase activity. In our investigation, we

took advantage of our recent observation [6] that SDS at low concentrations allowed the resolution of calcineurin subunits and importantly the resolved A subunit retained the phosphatase activity. In this communication, we describe the results of the influence of subunit B on the effects of calmodulin and divalent metals on subunit A phosphatase. We show that there is marked stimulation by the B subunit of  $Mn^{2+}$  stimulated protein phosphatase activity of subunit A. These results further support a critical role of trace metals like  $Mn^{2+}$  in calcineurin phosphatase activity [5–7].

## 2. EXPERIMENTAL

### 2.1. Materials

Bovine brain calmodulin was prepared by phenyl-Sepharose chromatography as in [10] and was further purified by DEAE-cellulose and Sephadex G-100 chromatography as described in [1]. Calcineurin was purified from bovine cerebral cortex as described in [5,6]. The enzyme catalyzed

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dephosphorylation of  $^{32}\text{P}$ -labelled myelin basic protein at a rate of 15–20 nmol  $^{32}\text{P}_i$ /min per mg in the presence of  $\text{Ca}^{2+}$  and calmodulin. One unit of enzyme is defined as the amount of enzyme that dephosphorylates 1 nmol/min. Purified myelin basic protein (MBP) from porcine brain (kindly provided by Eli Lilly) was phosphorylated as described in [5,6]; routinely,  $^{32}\text{P}$  incorporation amounted to 0.25–0.30 mol/mol MBP.  $\text{MgCl}_2$  and  $\text{CaCl}_2$  were purchased from Anachemia Chemicals, Toronto, and dialysis tubing and  $\text{MnCl}_2$  were obtained from Fisher Scientific. Dialysis tubing was treated, prior to use, by boiling in distilled water containing 10 mM EDTA for  $\frac{1}{2}$  h. After treatment, tubing was washed thoroughly with distilled water and stored at  $4^\circ\text{C}$  in the presence of distilled water containing 0.02%  $\text{NaN}_3$  and 1 mM EDTA. Tris base, catalytic subunit of cAMP dependent protein kinase (type II from bovine heart), bovine serum albumin,  $\text{NiCl}_2$ , dithiothreitol (DTT) and EGTA were obtained from Sigma. Phenyl Sepharose, activated cynogen bromide and Sephadex G-100 were from Pharmacia, and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was from New England Nuclear. All other reagents were of analytical grade.

## 2.2. Methods

### 2.2.1. Phosphatase assay

The phosphatase activity of the calcineurin holoenzyme was determined as described in [5,6]. Standard incubation contained 50 mM Tris-Cl, pH 7.0, 1 mg/ml BSA, 100 mM NaCl, 0.5 mM DTT, and concentrations of substrate, calcineurin or subunits of calcineurin, calmodulin and divalent cations as indicated in the figure and table legends. The release of  $^{32}\text{P}_i$  from phosphorylated substrate was linear with time and enzyme concentrations used in this work. Calcineurin or subunits of calcineurin (see below) were diluted appropriately with Tris-BSA buffer (50 mM Tris-Cl, pH 7.4, containing 1 mg/ml BSA) to maintain linear rate of dephosphorylation. Activities are expressed as nmol  $^{32}\text{P}_i$  released/min per mg of added calcineurin or subunit A.

We have noticed that there is variation in phosphatase specific activity amongst calcineurin preparations. The results described in this paper were seen using the same preparation of calci-

neurin. An unavoidable variability in the phosphatase activity results from the differences in the time of storage at  $-20^\circ\text{C}$  prior to use.

### 2.2.2. Preparation of subunits of calcineurin

Calcineurin was dialyzed against 50 mM Tris-Cl, pH 7.0, and stored at  $-20^\circ\text{C}$ . For the preparation of subunits, calcineurin was lyophilized and then dissolved in 500  $\mu\text{l}$  buffer A (50 mM Tris-Cl, pH 7.4, 5 mM DTT, 1 mM EGTA and 0.05% SDS). The resulting solution was applied to a Sephadex G-100 column ( $0.5 \times 115$  cm), pre-equilibrated with buffer A. The protein was eluted by buffer A and fractions of 0.5 ml were collected at a flow rate of 4–5 ml/h. Each fraction was analyzed for (a) protein content and (b) phosphatase activity; these fractions were also subjected to SDS-polyacrylamide gel electrophoresis.

### 2.2.3. Electrophoresis

Gel electrophoresis in the presence of 0.1% SDS was carried out on mini slab gel containing 12% acrylamide by Laemmli's method [11]. Proteins were stained with Coomassie brilliant blue and destained by acetic acid.  $M_r$  standards were run simultaneously.

### 2.2.4. Protein determination

Protein was determined by the Bio-Rad micro-assay procedure. BSA was used as a standard.

## 3. RESULTS

### 3.1. Resolution of calcineurin subunits by SDS/Sephadex G-100 chromatography

Calcineurin was dissociated into subunits A and B in the presence of 0.05% SDS and the dissociated subunits were resolved by molecular sieve chromatography on Sephadex G-100. The column was equilibrated in and eluted by buffer A containing 0.05% SDS. Subunit A was eluted in fractions 63–79 and subunit B, in fractions 89–99 (fig.1, inset). The phosphatase activity was detected in subunit A containing fractions and was not detected in fractions containing subunit B (fig.1). Minor bands of  $M_r$  43 000 and 32 000 were present in the subunit A containing fractions, which may be degradative products of the major band comprising subunit A ( $M_r$  61 000). It is important to recognize that polypeptide bands of  $M_r$  close to the

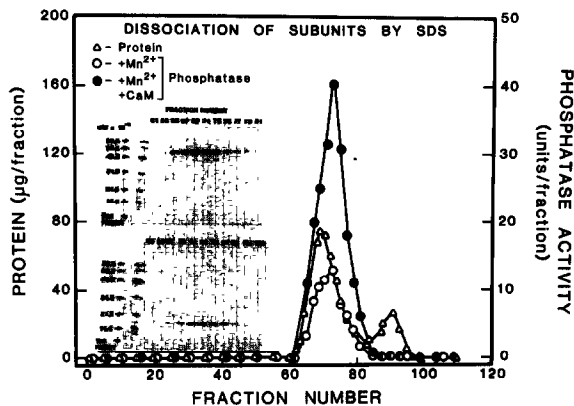


Fig.1. Resolution of calcineurin subunits by Sephadex G-100 column chromatography. The procedure for the separation of calcineurin subunits is described in section 2. For the assay of phosphatase activity, fractions were diluted 10-fold by Tris-BSA buffer and then an aliquot of 20  $\mu$ l was assayed for enzyme activity in a final volume of 50  $\mu$ l. Assay medium contained 50 mM Tris-HCl, pH 7.0, 1 mM EGTA, 100 mM NaCl, 0.5 mM DTT, 1 mg/ml BSA, and 50 pmol  $^{32}$ P-labelled MBP (1520 cpm/pmol) and one of the following additions: (1) none, (2) 2.2 mM  $\text{CaCl}_2$ , (3) 2.2 mM  $\text{MnCl}_2$ , (4) 2.2 mM  $\text{NiCl}_2$ , (5) 2.2 mM  $\text{CaCl}_2$  + 1  $\mu$ M calmodulin (CaM), (6) 2.2 mM  $\text{MnCl}_2$  + 1  $\mu$ M CaM, and (7) 2.2 mM  $\text{NiCl}_2$  + 1  $\mu$ M CaM. Reaction was started by adding  $^{32}$ P-labelled MBP and was carried out at 33°C for 10 min. The phosphatase activity was not detected when assayed under the conditions 1, 2, 4, 5 and 7 (not shown). Enzyme activity was detectable in the presence of  $\text{Mn}^{2+}$  (○) and  $\text{Mn}^{2+}$  + CaM (●). Separate experiments established that amount of SDS carried over (0.002%) from the column fractions did not inhibit the enzyme activity. The inset shows SDS-PAGE electrophoretic patterns of eluted fractions. 20  $\mu$ l fraction was mixed with 10  $\mu$ l of Laemmli's digester and of this 10  $\mu$ l was applied to 12% SDS mini slab gel. Electrophoresis was carried out as described in section 2. Number on the top of the gel corresponds to the number of eluted fractions.

subunit B were absent in the fractions 65-79 (subunit A fractions), i.e. subunit A was eluted free of subunit B. These results clearly document that protein phosphatase (assayed with  $^{32}$ P-myelin basic protein as substrate) resides exclusively in subunit A and confirm our previous result [6] seen following electrophoretic separation of calcineurin subunits.

### 3.2. $\text{Mn}^{2+}$ is required for subunit A phosphatase activity

The fractions containing subunit A (67-71) and subunit B (89-97) were pooled and concentrated. The phosphatase activity of either fraction was determined in the presence of various divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Ni}^{2+}$ ) without and with calmodulin in the assay mixture. The B subunit fractions showed no enzyme activity under all assay conditions (see fig.2). The phosphatase activity of subunit A was detected only in the presence of  $\text{Mn}^{2+}$  (fig.1); calmodulin stimulated  $\text{Mn}^{2+}$ -dependent activity 4-5-fold (see fig.1).

It was surprising and unexpected that the A subunit phosphatase activity, even when assayed with calmodulin present, was not supported by  $\text{Ca}^{2+}$  or  $\text{Ni}^{2+}$ . Such observations further point out

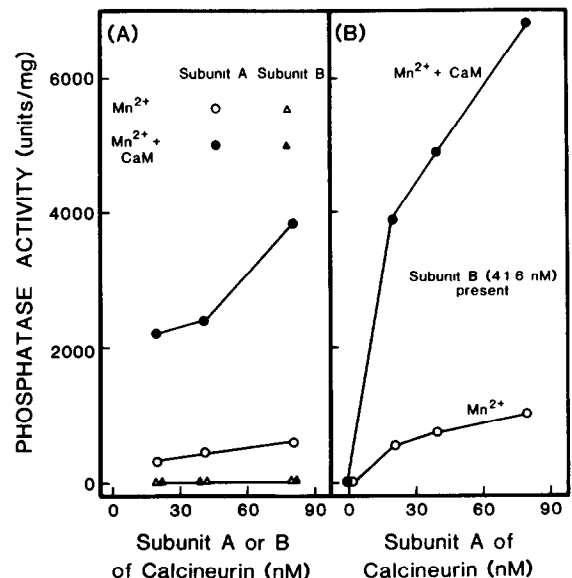


Fig.2. Effect of subunit B on subunit A phosphatase activity. (Left) Subunits A or B obtained from gel filtration column were appropriately diluted by Tris-BSA buffer and phosphatase activity was assayed in 60  $\mu$ l reaction mixture containing 50 mM Tris-HCl, pH 7.0, 1 mg/ml BSA, 100 mM NaCl, 0.5 mM DTT, and 100 pmol  $^{32}$ P-labelled MBP. When present  $\text{MnCl}_2$  was 1.1 mM and calmodulin (CaM), 1  $\mu$ M. (Right) Phosphatase activity was assayed at varying concentrations (0-80 nM) of subunit A in the presence of fixed concentration of subunit B (41.6 nM). The assay was carried out in the presence of 1.1 mM  $\text{MnCl}_2$  and with and without CaM (1  $\mu$ M).

Table 1

Effect of dialysis on subunit A associated phosphatase activity assayed in the presence and absence of subunit B

Additions	Subunit A phosphatase (units/mg)			
	Undialyzed		Dialyzed	
	- Subunit B	+ Subunit B	- Subunit B	+ Subunit B
Mn <sup>2+</sup>	3.16	3.88	0.33	0.41
Mn <sup>2+</sup> + CaM	15.52	31.77	1.86	2.22

Subunits A and B of calcineurin (dissociated by SDS and separated on Sephadex G-100 column) were dialyzed against 4 l of 25 mM Tris-Cl, pH 7.5, at 4°C for 18 h. Phosphatase activity was assayed in a 60 µl reaction volume that contained 50 mM Tris-Cl, pH 7.0, 1 mM EGTA, 1 mg/ml BSA, 100 mM NaCl, 0.5 mM DTT, 50 pmol <sup>32</sup>P-labelled myelin basic protein (1484 cpm/pmol) and 0.42 µM subunit A or B or both (of dialyzed or undialyzed preparations). When present, MnCl<sub>2</sub> was 1.1 mM and calmodulin (CaM) was 1 µM. Note that no activity was detected when subunit A was assayed in the absence of divalent cations or presence of CaCl<sub>2</sub> or NiCl<sub>2</sub> with and without CaM (not shown)

a crucial role of Mn<sup>2+</sup> in supporting calcineurin phosphatase [5-7] and particularly in the phosphatase expressed by the resolved A subunit.

### 3.3. Influence of subunit B on subunit A phosphatase

As was mentioned earlier, the resolved B subunit lacked the phosphatase activity (fig.2, left panel). This allowed one to test whether this subunit exerts any effect on the subunit A phosphatase activity. Fig.2 shows a few interesting features of the subunit A-B interaction. First, addition of subunit B modestly augmented the Mn<sup>2+</sup>-dependent phosphatase of subunit A. This increase, however, was found to be marked especially when the phosphatase was assayed with Mn<sup>2+</sup> and calmodulin present (fig.2, left panel). For example, in the absence of subunit B, the specific activity was 23 units/mg subunit A and this was increased to 48 units/mg subunit A when equimolar amounts (41.6 nM) of A and B units were present in the assay (fig.2, right panel). These results suggest that B subunit somehow augmented the stimulatory effect of calmodulin on the Mn<sup>2+</sup>-dependent phosphatase of subunit A. In preliminary work, the maximum stimulation by subunit B of subunit A (5 nM) phosphatase activity (assayed with Mn<sup>2+</sup> and calmodulin) was seen in the presence of about 20 nM subunit B.

### 3.4. Lability of resolved A subunit phosphatase

In 3 trials, we observed that the phosphatase activity of A subunit was readily detectable in the presence of Mn<sup>2+</sup> for 3 days following its isolation by SDS/Sephadex G-100 chromatography. This activity, however, declined steadily over the 3 day period when the resolved A subunit was stored at 4°C. Since SDS was present, we attempted to dialyze out this detergent (at 4°C); however, this was always associated with marked losses in the phosphatase activity assayed with Mn<sup>2+</sup> ± calmodulin (table 1). In preliminary work, we noted slightly improved preservation of the phosphatase activity when pooled fractions were stored frozen at -20°C. The results shown in this work were carried out within 48 h after the separation of the calcineurin subunits by SDS/Sephadex G-100 chromatography. While the specific activities of the resolved A subunit varied with the batch of calcineurin, the effects of divalent cations and calmodulin were qualitatively similar to those described in this communication.

## 4. DISCUSSION

We describe here the dissociation of calcineurin subunits by SDS and their resolution by molecular sieve chromatography in the presence of SDS. The

results showed the presence of phosphatase activity in the A subunit fractions. Previously we observed the presence of phosphatase activity in the A subunit, but not in the B subunit, of calcineurin fractionated by electrophoresis [6]. The present work made it possible to investigate a few major aspects of calcineurin. For example, we could test whether divalent metals like  $Mn^{2+}$  and calmodulin regulate the calcineurin phosphatase activity by direct action on the A subunit. The results clearly showed this to be the case. To date, the role of B subunit has remained unclear (e.g. [8]). In this study we found that this subunit augmented calmodulin effect on subunit A phosphatase. Calcineurin B subunit and calmodulin reveal considerable homology [12] and yet B subunit, which has 4  $Ca^{2+}$  binding sites [1], did not influence the activity of subunit A phosphatase assayed with  $Ca^{2+}$ . Further, calmodulin with  $Ca^{2+}$  also failed to exert any effect on subunit A phosphatase. Instead,  $Mn^{2+}$  was required. The phosphatase activity of holoenzyme is increased by  $Ca^{2+}$  in the presence of calmodulin [2-9] and also by  $Mn^{2+}$  ( $\pm$  calmodulin) [5-7]. Thus, although we have seen the stimulation by the B subunit of  $Mn^{2+}$ /calmodulin augmented subunit A phosphatase, it appears that in the process of isolation of these subunits we have modified certain features (e.g. lack of  $Ca^{2+}$  or  $Ni^{2+}$  effects) that are evident in the holoenzyme. In this context the lack of stimulation by  $Ni^{2+}$  is noteworthy, since like  $Mn^{2+}$ , this cation is believed to interact with subunit A [8,13].

Recently, Merat et al. [14] in an abstract reported the resolution of the calcineurin subunits by gel filtration in 6 M urea. Their results showed the stimulation of subunit A phosphatase by subunit B or by calmodulin in the presence of  $Ca^{2+}$ . This indicates that SDS-dependent (our study) and urea-dependent [14] resolution of calcineurin subunits modifies, perhaps in a subtle way, the divalent cation dependence of the action of calmodulin and subunit B on the phosphatase activity of subunit

A. We are currently pursuing investigation of this issue, since the results will provide important clues about the divalent metal regulation of this critical enzyme.

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